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Activating Transcription Factor 6 Polymorphisms and Haplotypes Are Associated with Impaired Glucose Homeostasis and Type 2 Diabetes in Dutch Caucasians

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Context: Activating transcription factor 6 (*ATF6*) is critical for initiation and full activation of the unfolded protein response. An association between genetic variation in *ATF6* and type 2 diabetes (DM2) was recently reported in Pima Indians.

Objectives: To investigate the broader significance of this association for DM2, replication studies in distinct ethnic populations are required. We investigated *ATF6* for its association with DM2 in Dutch Caucasians.

Design/Setting: A genetic association study was conducted at an academic research laboratory.

Study Participants: Two independent Dutch cohorts were studied. Cohort 1 (*n* = 154) was used to evaluate genetic variation in the *ATF6* gene in relation to glucose homeostasis in the general population. Cohort 2 (*n* = 798) consisted of patients with DM2, impaired glucose tolerance, impaired fasting glucose, and normoglycemic control sub-

jects, and was used to investigate *ATF6* polymorphisms for their contribution to disturbed glucose homeostasis and DM2.

Main Outcome Measures: There were 16 tag single nucleotide polymorphisms genotyped in all subjects of both cohorts. Those single nucleotide polymorphisms included three nonsynonymous coding variants and captured all common allelic variation of *ATF6*.

Results: Our data show that common *ATF6* variants are associated with elevated glucose levels in the general population (cohort 1, *P* = 0.005–0.05). Furthermore, the majority of these variants, and haplotypes thereof, were significantly associated with impaired fasting glucose, impaired glucose tolerance, and DM2 (cohort 2, *P* = 0.006–0.05). Associated variants differ from those identified in Pima Indians.

Conclusions: Our results strengthen the evidence that one or more variants in *ATF6* are associated with disturbed glucose homeostasis and DM2. (*J Clin Endocrinol Metab* 92: 2720–2725, 2007)

EVIDENCE IS EMERGING in literature that the endoplasmic reticulum (ER) may represent a major link between obesity, insulin resistance, β -cell function, and type 2 diabetes (DM2) (1–5). Obesity generates stress conditions that increase the demand on the ER. To cope with this stress, cells initiate a signal transduction pathway called the ER-stress response, or unfolded protein response (UPR). Activation of the UPR is regulated by three ER stress sensors:

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Abbreviations: *ATF6*, Activating transcription factor 6; BMI, body mass index; DM2, type 2 diabetes; ER, endoplasmic reticulum; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; IRE1, inositol requiring 1; MAF, minor allele frequency; PERK, protein kinase R-like ER kinase; SNP, single nucleotide polymorphism; UPR, unfolded protein response.

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protein kinase R-like ER kinase (PERK), inositol requiring 1 (IRE1), and activating transcription factor 6 (*ATF6*) (6). Activation of PERK, IRE1, and *ATF6* is under control of a master UPR regulator, the ER chaperone protein BiP/Grp78. The PERK and IRE1 initiated pathways of the UPR play an important role in DM2 through effects on β -cell function and peripheral insulin signaling, respectively (1, 3).

Within the framework of a previous gene expression study (7), we found a positive significant correlation between *in vitro* mRNA levels of BiP/Grp78 in cultured Epstein-Barr virus-transformed lymphoblasts and the fasting plasma glucose levels that had been measured in the corresponding subjects, *in vivo*. Therefore, we hypothesized that the intrinsic cellular UPR capacity may partially determine a subject's plasma glucose level and, moreover, that genetic variation in UPR genes may contribute to DM2. An excellent candidate gene to link UPR capacity to DM2 is *ATF6*. First, *ATF6* is

essential for full activation of the UPR and considered as a critical UPR initiator in mammalian cells (8). Second, *ATF6* induces expression of BiP/Grp78 both directly and indirectly via activation of X-box binding protein 1. Third, *ATF6* maps to chromosome 1q23.3, the most replicated chromosomal locus for DM2 (9). While preparing this manuscript, Thameem *et al.* (10) reported an association of *ATF6* with DM2 in Pima Indians. To investigate the broader significance of this association for DM2, replication studies in additional populations are required. In the present study, we investigated *ATF6* as a positional and functional candidate gene for disturbed glucose homeostasis and DM2.

Subjects and Methods

Study populations

Two independent Dutch cohorts, comprising a total of 952 Caucasian subjects, were used to investigate the genetic association between polymorphisms in the *ATF6* gene, disturbed glucose homeostasis, and DM2. The first cohort ($n = 154$) was recruited as spouses of hyperlipidemic patients, collected within the framework of a family study on the genetics of familial combined hyperlipidemia. These subjects, not selected in any way, represented the general population and exhibited the following clinical characteristics: 42% males, age 51 ± 11 yr, body mass index (BMI) 25.4 ± 3.9 kg/m², fasting blood glucose 4.9 ± 0.7 mmol/liter, fasting plasma insulin 6.1 ± 5.4 μ U/ml, and homeostasis model of assessment insulin resistance 1.4 ± 1.5 . In this cohort, 137 subjects (89%) had normal fasting glucose levels, 16 subjects (10.5%) had impaired fasting glucose (IFG) levels, and one subject (<1%) had DM2. No DNA was available for three subjects, leaving 151 subjects available for analyses. This cohort was used to explore the genetic variation in the *ATF6* gene in relation to glucose homeostasis in the general population.

A second independent Dutch cohort ($n = 798$) that consisted of 211 DM2 patients, 208 IFG/impaired glucose tolerance (IGT) subjects, and 379 normoglycemic controls was used to investigate *ATF6* polymorphisms for their contribution to disturbed glucose homeostasis and DM2. This case control cohort was collected as described previously (11, 12). Briefly, more than 2700 subjects with one or more cardiovascular risk factors, including hypertension, BMI more than 25 kg/m², a positive family history for DM2, or a history of gestational diabetes, were screened for DM2. Exclusion criteria were the use of medication that affects glucose metabolism and non-Caucasian ethnicity. All newly diagnosed subjects with IFG, IGT, or DM2, and a random selection of 379 normoglycemic control subjects were included in the present study. Characteristics of this cohort are provided in Table 1. The Human Investigation Review Committee of the Academic Hospital Maastricht approved the study protocol, and all subjects gave written informed consent.

Single nucleotide polymorphism (SNP) selection and genotyping

SNP selection was designed to achieve 100% coverage of the common genetic variation [minor allele frequency (MAF) > 10%] of the *ATF6* gene, including 3-kb upstream and downstream this locus. Using the HapMap database (<http://www.hapmap.org/index.html.en>), 13 tag SNPs were selected for genotyping. One tag SNP (SNP4) was located in an exon and caused an amino acid substitution. In addition to the selection of 13 SNPs by the tagging process, three additional SNPs were selected for their potential direct functional effect: SNP3 and SNP5 for

their location in exons, causing an amino acid substitution, and SNP15 for its location in the 3' untranslated region. In total, 16 SNPs were genotyped in 952 subjects. Genotyping was done using the TaqMan 7900HT (Applied Biosystems, Foster City, CA).

Statistical analyses

Power calculations were performed using the genetic power calculator of Purcell *et al.* (13) (<http://pngu.mgh.harvard.edu/~purcell/gpc/>). Assuming a disease prevalence of 0.1, a genotype relative risk of 1.1 and 2.3 [the calculated relative risks of Aa and AA, respectively, in a previous study (10)], and an allele frequency of 0.2 [the frequency of the *ATF6* risk allele, previously associated with DM2 (10)], the case control sample afforded an estimated power of 71% at $P < 0.05$. Linkage disequilibrium statistics were analyzed by Haploview software (14). Haplotypes were constructed using the program SNPHAP (<http://www-gene.cimr.cam.ac.uk/clayton/software>). Analyses of variance and Student's *t* tests were used to test polymorphisms in the *ATF6* gene for association with plasma glucose levels. Odds ratios and *P* values for case control analyses were calculated using logistic regression analyses in which "allele" was parameterized in the model as a dichotomous variable. Adjustments for age, gender, and BMI were done by including these variables in the logistic regression model.

Results

The intrinsic capacity of the UPR may influence fasting plasma glucose levels

In a previous gene expression experiment, designed to study the genetic background of combined hyperlipidemia, we cultured Epstein-Barr virus-transformed lymphoblast cells from 12 hyperlipidemic patients and 12 normolipidemic controls (7). To explore further this data set in relation to glucose homeostasis, we measured plasma glucose levels in these subjects and reanalyzed transcript levels in relation to this previously unstudied trait. One subject with a fasting plasma glucose more than 3 sds of the mean was excluded, leaving 23 subjects for analyses. Subjects exhibited the following characteristics: age 50 ± 9 yr, 11 males/12 females, and fasting plasma glucose levels 5.3 ± 0.8 mmol/liter. A complete clinical characterization of these subjects has been provided in an article by Morello *et al.* (7).

We observed a strong positive correlation ($r = 0.57$; $P = 0.004$) between *in vitro* mRNA levels of BiP/Grp78 and the fasting plasma glucose levels in the corresponding subjects, *in vivo*. This correlation is very unlikely the result of the well-known response of BiP/Grp78 expression to glucose (15) because all cells had been cultured for several weeks under isoglycemic and standardized conditions. This observation led to the hypothesis that the intrinsic UPR capacity may partially determine fasting plasma glucose levels and that UPR genes may be involved in glucose homeostasis. Therefore, we performed a genetic association study with *ATF6*, a key gene of the UPR, and transcriptional regulator of BiP/Grp78.

Genetic variation in the *ATF6* gene

The *ATF6* gene consists of 16 exons that span approximately 193-kb genomic DNA. The gene structure of *ATF6*, the frequencies of the minor alleles for each SNP, and the pairwise correlation of alleles between various SNPs are depicted in Fig. 1 [data for cohort 2 ($n = 798$)]. Genotype

TABLE 1. Characteristics of cohort 2

Trait	NGT	IFG/IGT	DM2
No. (male/female)	379 (222/157)	208 (125/83)	211 (144/67)
Age (yr)	58 ± 7	58 ± 7	60 ± 7
BMI (kg/m ²)	27.4 ± 3.8	29.3 ± 4.2	30.7 ± 4.4

Data are means \pm SD, unless otherwise indicated. NGT, Normal glucose tolerance.

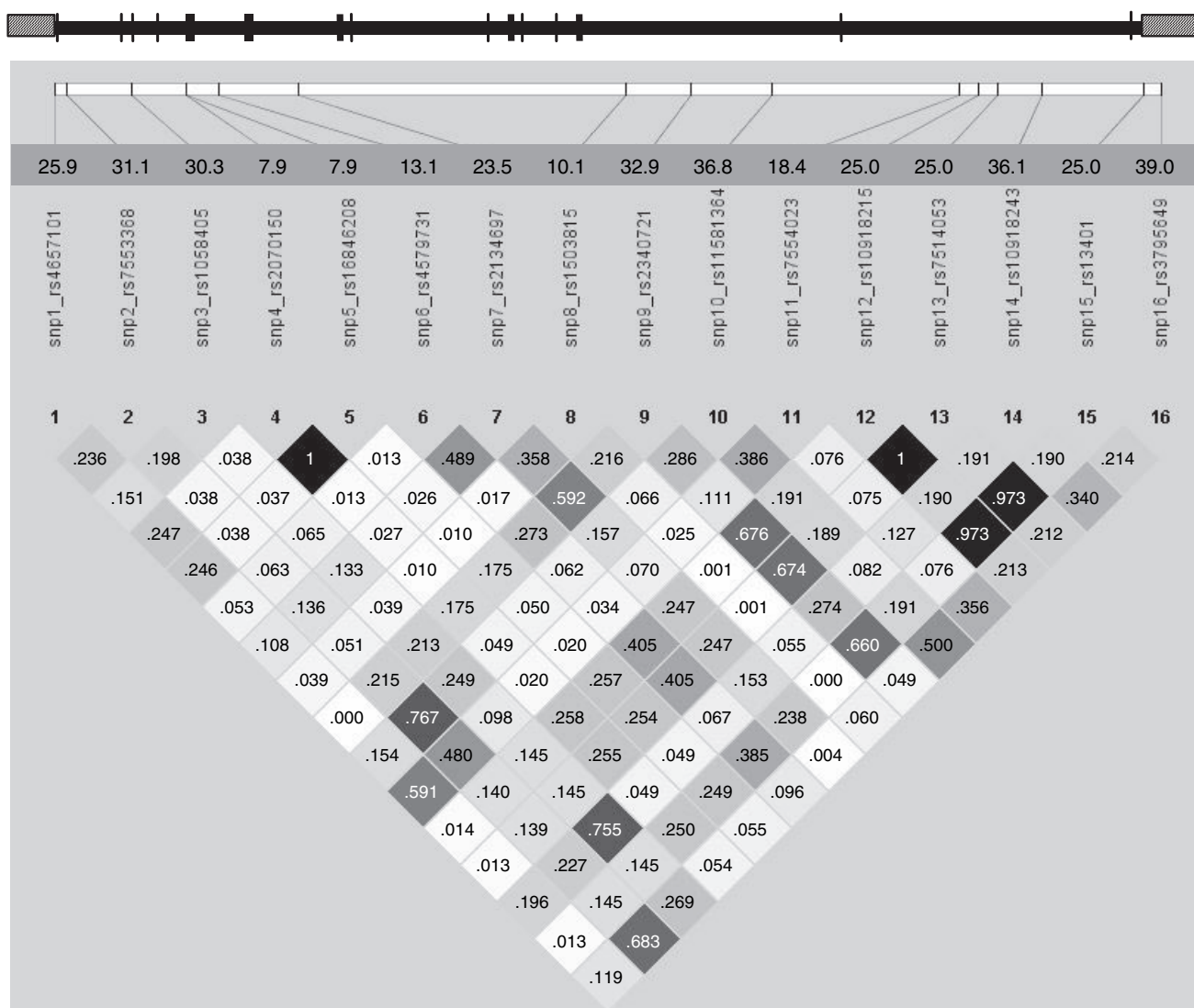


FIG. 1. Genetic structure of the *ATF6* gene, including the 16 genotyped SNPs and MAFs. Dashed boxes represent the promoter region and the 3' untranslated region. Exons are represented by vertical lines. MAFs of the genotyped SNPs are shown in the gray horizontal bar. The shade of the diamonds represents the pairwise r^2 between the two SNPs defined by the top left and the top right sides of the diamond. Shading represents the magnitude of pairwise r^2 , with at the extremes white reflecting very low r^2 (<0.20) and black reflecting very high r^2 (>0.80).

success rate was more than 97% for all 16 genotyped SNPs. All SNPs were in Hardy-Weinberg equilibrium.

ATF6 polymorphisms are associated with fasting plasma glucose levels in the general population (cohort 1)

First, we investigated whether genetic variation in the *ATF6* gene was associated with fasting glucose levels in the first cohort from the general population (Table 2). ANOVAs revealed a significant association of SNPs 9, 12, 13, and 15 with fasting glucose levels in this cohort ($P = 0.018$ – 0.053). Further analyses under a dominant genetic model strengthened the evidence for association with these SNPs ($P = 0.005$ – 0.015). In addition, SNP3 and SNP6 were borderline significant under a dominant genetic model. These data suggest that *ATF6* polymorphisms modulate plasma glucose levels in the general population.

ATF6 polymorphisms are associated with disturbed glucose homeostasis and DM2 (cohort 2)

Next, to test the hypothesis that variation in *ATF6* contributes to disturbed glucose metabolism and DM2, we genotyped all selected SNPs in the second cohort ($n = 798$), comprising 211 patients with DM2, 208 subjects with IFG/IGT, and 379 normoglycemic controls (Table 3). In line with results from the first cohort, prevalence of the minor allele of SNPs 12, 13, and 15 was significantly increased in cases compared with control subjects ($P = 0.033$ – 0.051). Furthermore, SNP6, which showed a trend for association in cohort 1, was significantly different in cases and controls of cohort 2 (unadjusted $P = 0.027$). For SNP3 and SNP9, we were not able to confirm the significant association of cohort 1. Statistical analyses using alternative genetic models (genotype, dominant, and recessive) support these findings ($P = 0.01$ –

TABLE 2. Associations (genotype effects) of ATF6 polymorphisms with fasting plasma glucose levels in the general population (cohort 1)

SNP	Genotype	n	Glucose \pm SD (mmol/liter)	<i>P</i> value genotype	<i>P</i> value recessive	<i>P</i> value dominant
SNP 1 (rs 4657101)	A/A	94	4.85 \pm 0.54	0.501	0.938	0.276
	A/C	34	5.00 \pm 0.89			
	C/C	12	4.90 \pm 0.51			
SNP 2 (rs 7553368)	T/T	70	4.95 \pm 0.68	0.789	0.559	0.584
	T/C	56	4.90 \pm 0.73			
	C/C	16	4.82 \pm 0.61			
SNP 3 (rs 1058405)	A/A	78	5.00 \pm 0.79	0.123	0.331	0.043
	A/G	53	4.78 \pm 0.57			
	G/G	13	4.72 \pm 0.45			
SNP 4 (rs 2070150)	G/G	125	4.88 \pm 0.65	0.357	0.297	0.232
	G/C	17	5.05 \pm 0.81			
	C/C	1	5.60			
SNP 5 (rs 16846208)	C/C	124	4.89 \pm 0.67	0.356	0.323	0.214
	C/T	16	5.08 \pm 0.83			
	T/T	1	5.6			
SNP 6 (rs 4579731)	A/A	102	4.84 \pm 0.58	0.127	0.657	0.057
	A/G	38	5.10 \pm 0.91			
	G/G	1	4.60			
SNP 7 (rs 2134697)	T/T	76	4.81 \pm 0.58	0.306	0.625	0.125
	T/C	63	4.99 \pm 0.83			
	C/C	6	5.03 \pm 0.45			
SNP 8 (rs 1503815)	C/C	115	4.89 \pm 0.72	0.838	0.552	0.912
	C/T	31	4.89 \pm 0.50			
	T/T	1	5.3			
SNP 9 (rs 2340721)	A/A	61	4.73 \pm 0.51	0.033	0.567	0.009
	C/A	73	5.03 \pm 0.77			
	C/C	14	5.01 \pm 0.75			
SNP 10 (rs 11581364)	G/G	53	4.93 \pm 0.59	0.857	0.664	0.636
	G/T	66	4.89 \pm 0.69			
	T/T	22	4.84 \pm 0.54			
SNP 11 (rs 7554023)	A/A	107	4.94 \pm 0.65	0.630	0.397	0.441
	A/G	28	4.87 \pm 0.88			
	G/G	8	4.71 \pm 0.29			
SNP 12 (rs 10918215)	A/A	80	4.77 \pm 0.53	0.025	0.390	0.006
	A/G	56	5.08 \pm 0.81			
	G/G	10	5.09 \pm 0.83			
SNP 13 (rs 7514053)	G/G	78	4.76 \pm 0.53	0.018	0.376	0.005
	G/A	56	5.08 \pm 0.81			
	A/A	10	5.09 \pm 0.83			
SNP 14 (rs 10918243)	T/T	64	4.98 \pm 0.76	0.260	0.120	0.304
	T/C	61	4.91 \pm 0.65			
	C/C	18	4.68 \pm 0.48			
SNP 15 (rs 13401)	A/A	82	4.77 \pm 0.53	0.053	0.577	0.015
	A/G	57	5.05 \pm 0.82			
	G/G	12	5.00 \pm 0.79			
SNP 16 (rs 3795649)	C/C	57	4.94 \pm 0.73	0.849	0.616	0.657
	C/T	62	4.91 \pm 0.66			
	T/T	26	4.85 \pm 0.67			

P values in *bold* reflect statistical significance.

0.05) (supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>).

Haplotype analysis

Haplotype analysis provided similar results. Statistical reconstruction of haplotypes comprising the four significantly associated SNPs (6, 12, 13, and 15) identified three common haplotypes, which together accounted for 99% of observed haplotypes in cases and controls (Table 4). We identified an "at risk" haplotype (GGAG), with higher prevalence in subjects with DM2, IGT, and IFG compared with controls [14.4% *vs.* 9.9%; odds ratio 1.54; 95% confidence interval 1.13–2.09; *P* = 0.006]. The opposite AAGA haplotype showed a trend for higher prevalence in control subjects compared with sub-

jects with disturbed glucose homeostasis (76.4% *vs.* 72.2%; odds ratio 0.80; 95% confidence interval 0.64–1.01; *P* = 0.056). It is noteworthy that stratified analyses showed that the prevalence of the GGAG-risk haplotype was significantly increased in both subjects with IFG or IGT (odds ratio 1.59; *P* = 0.011) as well as in patients with fully developed DM2 (odds ratio 1.48; *P* = 0.036).

Discussion

ATF6 is essential for full activation of the UPR and considered a critical UPR initiator in mammalian cells (8). In the present study, we show that *ATF6* polymorphisms and haplotypes are associated with disturbed glucose homeostasis and DM2 in Dutch Caucasians. While this work was in progress, a genetic association between amino acid variants

TABLE 3. ATF6 SNP allele frequencies in cases (DM2, IFG, and IGT) and normoglycemic control subjects (cohort 2)

SNP rs no.	No. of alleles	Affected status	No. of allele 1 (%)	No. of allele 2 (%)	<i>P</i> value	<i>P</i> value adjusted ^a
SNP 1 (rs 4657101)	1570	Controls	551 (0.74)	197 (0.26)	0.680	0.805
		Cases	613 (0.75)	209 (0.25)		
SNP 2 (rs 7553368)	1570	Controls	506 (0.68)	244 (0.32)	0.235	0.237
		Cases	576 (0.70)	244 (0.30)		
SNP 3 (rs 1058405)	1582	Controls	527 (0.70)	227 (0.30)	0.887	0.855
		Cases	576 (0.70)	252 (0.30)		
SNP 4 (rs 2070150)	1578	Controls	693 (0.92)	61 (0.08)	0.812	0.974
		Cases	760 (0.92)	64 (0.08)		
SNP 5 (rs 16846208)	1584	Controls	693 (0.92)	61 (0.08)	0.780	0.992
		Cases	766 (0.92)	64 (0.08)		
SNP 6 (rs 4579731)	1564	Controls	656 (0.89)	82 (0.11)	0.027	0.037
		Cases	703 (0.85)	123 (0.15)		
SNP 7 (rs 2134697)	1576	Controls	590 (0.79)	162 (0.21)	0.074	0.082
		Cases	615 (0.75)	209 (0.25)		
SNP 8 (rs 1503815)	1566	Controls	672 (0.90)	76 (0.10)	0.929	0.985
		Cases	736 (0.90)	82 (0.10)		
SNP 9 (rs 2340721)	1584	Controls	519 (0.69)	235 (0.31)	0.180	0.136
		Cases	545 (0.66)	285 (0.34)		
SNP 10 (rs 11581364)	1574	Controls	458 (0.61)	290 (0.39)	0.133	0.165
		Cases	536 (0.65)	290 (0.35)		
SNP 11 (rs 7554023)	1574	Controls	608 (0.81)	140 (0.19)	0.729	0.730
		Cases	677 (0.82)	149 (0.18)		
SNP 12 (rs 10918215)	1578	Controls	580 (0.77)	170 (0.23)	0.039	0.043
		Cases	603 (0.73)	225 (0.27)		
SNP 13 (rs 7514053)	1592	Controls	575 (0.77)	169 (0.23)	0.051	0.058
		Cases	606 (0.73)	242 (0.27)		
SNP 14 (rs 10918243)	1576	Controls	481 (0.64)	273 (0.36)	0.935	0.758
		Cases	526 (0.64)	296 (0.36)		
SNP 15 (rs 13401)	1596	Controls	586 (0.77)	172 (0.23)	0.033	0.042
		Cases	609 (0.73)	229 (0.27)		
SNP 16 (rs 3795649)	1570	Controls	439 (0.59)	307 (0.41)	0.084	0.143
		Cases	520 (0.63)	304 (0.37)		

P values in **bold** reflect statistical significance.

^a Adjusted for age, gender, and BMI.

in *ATF6* and DM2 was reported in Pima Indians (10). Additional replication studies are strongly required to validate these findings and evaluate their broader significance for predisposition to DM2 in different ethnic populations.

In the present study, we identified polymorphisms in the *ATF6* gene, significantly associated with fasting glucose levels in a cohort representative for the general population, and with disturbed glucose homeostasis/DM2 in a second cohort, ascertained for IFG, IGT, and DM2. Allele frequencies from associated SNPs 12, 13, and 15 were slightly higher in subjects from the general population in cohort 1 (~26%) than in normoglycemic control subjects in cohort 2 (23%). Different recruitment criteria and statistical variance may account for this difference: control subjects in cohort 2 are nondiabetic, verified by an oral glucose tolerance test, whereas

subjects from the general population in cohort 1 may include some undetected DM2 patients.

The associated SNPs in this study were all located in non-coding regions of the *ATF6* gene. SNPs 6, 12, and 13 map to introns, whereas SNP15 locates to the 3' untranslated region. It is noteworthy that SNP15 is in direct vicinity (11 base pairs in 5' direction) of an AATAAA-poly-adenylation sequence in the 3' untranslated region. This recognition site is involved in polyadenosine tail formation, which protects the mRNA molecule from exonucleases, and is important for transcription termination, for export of the mRNA from the nucleus, and for translation.

Three known exonic variants have been described in *ATF6*: Pro[145]Ala, Ser[157]Pro, and Met[67]Val. In Pima Indians, the amino acid variants Pro[145]Ala and Ser[157]Pro polymorphism showed an association ($P = 0.05$) with DM2 (10). These amino acid substitutions (SNP4 and SNP5, respectively, in our study) were not significantly associated with fasting glucose levels in our first cohort, or with disturbed glucose homeostasis and DM2 in the second cohort. The negative result is very unlikely due to a lack of statistical power because allele frequencies were identical in cases and controls, not even revealing a trend toward significance (SNP4 adjusted $P = 0.97$; SNP5 adjusted $P = 0.99$). The third known *ATF6* amino acid variant, Met[67]Val (SNP3), which was not previously found associated with DM2 in Pima Indians (10), was associated with fasting plasma glucose

TABLE 4. ATF6 haplotype distribution in cases (DM2, IFG, and IGT) and normoglycemic control subjects

Haplotype	Control subjects (%)	Cases (%)	<i>P</i> value
AAGA	76.4	72.2	0.056
AGAG	12.5	12.6	0.944
GGAG	9.9	14.4	0.006
Rare	1.2	0.7	0.330
Total	100	100	

Haplotype frequencies for the four significantly associated SNPs in Table 2 were estimated with SNPHAP. Of haplotypes, 97% were estimated with a probability > 97%. *P* values in **bold** reflect statistical significance.

levels in cohort 1, but again, allele frequencies were not significantly different between cases and controls in cohort 2. Further genetic research and functional studies are needed to establish which SNP(s) in the *ATF6* gene are functionally responsible for the observed associations in both populations. Although we do not provide a formal replication of the genetic association in Pima Indians, in which replication is defined by same allele, same genetic model, and same phenotype, our data do, however, confirm and extend the nature of their observations, and emphasize that the *ATF6* gene is also relevant for susceptibility to disturbed glucose homeostasis and DM2 in Dutch Caucasians.

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